A proteomic study reveals novel insights into the diversity of aquaporin forms expressed in the plasma membrane of plant roots

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Aquaporins are channel proteins that facilitate the diffusion of water across cell membranes. The genome of *Arabidopsis thaliana* encodes 35 full-length aquaporin homologues. Thirteen of them belong to the plasma membrane intrinsic protein (PIP) subfamily and predominantly sit at the plasma membrane (PM). In the present work we combine separations of membrane proteins (by one- and two-dimensional gel electrophoresis) with identification by MS (matrix-assisted laser-desorption ionization–time-of-flight and electrospray-ionization tandem MS) to take an inventory of aquaporin isoforms expressed in the PM of *Arabidopsis thaliana* roots. Our analysis provides direct evidence for the expression of five PIPs (PIP1;1, PIP1;5, PIP2;1, PIP2;2 and PIP2;7) in the root PM and suggests the presence of at least three other PIP isoforms. In addition, we show that the same PIP isoform can be present under several forms with distinct isoelectric points. More specifically, we identify phosphorylated aquaporins in the PIP1 and PIP2 subgroups and suggest the existence of other post-translational modifications. Their identification should provide clues to reveal novel molecular mechanisms for aquaporin regulation.

Key words: aquaporin, mass spectrometry (MS), phosphorylation, plasma membrane, root, two-dimensional electrophoresis.

INTRODUCTION

Plants require a continuous uptake of water to sustain transpiration and growth. In roots, the radial transport of water across living tissues down to xylem vessels can occur through two parallel pathways, i.e. across cell walls or from cell to cell. The latter pathway is mediated in part by water-channel proteins, called aquaporins. Aquaporins facilitate the diffusion of water across cell membranes and thus play an important role in plant–water relations [1–3]. In particular, a critical contribution of aquaporins to short-term regulation of plant water status in response to various environmental factors (salinity, anoxia, nutrient deficiency and day/night cycles) has been proposed (see [2] for review).

Aquaporins are 26–35 kDa proteins that belong to the major intrinsic protein (MIP) superfamily and share a typical structure with six transmembrane α-helices linked by five loops (A–E). Their N- and C-termini are both located on the cytoplasmic side of the membrane. The aqueous pore is formed in part by two Asn-Pro-Ala ('NPA') motifs that are located in the connecting loops B and E, respectively [4,5].

Based upon their amino acid sequence homology, plant MIPs can be classified into four subfamilies [6–8]. The plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs) are located in the plasma membrane (PM) and the vacuolar membrane (tonoplast), respectively. The subcellular localization of members of the two other classes, the nodulin-26-like intrinsic proteins (NIPs) and the small basic intrinsic proteins ('SIPs'), is still unknown. The variety of cellular localizations and numerous cell- and organ-specific expression patterns of plant MIPs can in part explain their high diversity [9]. The genome of *Arabidopsis thaliana*, for instance, encodes 35 full-length aquaporin homologues. Thirteen of them belong to the PIP subfamily which can be divided further into two sequence homology groups, PIP1 and PIP2.

In recent years, aquaporin expression in plant roots has been the subject of several studies; however, these have been restricted to patterns of a few individual aquaporin genes (see [2] for review). In addition, immunodetection of specific aquaporin isoforms was made difficult by the high sequence homology between members of the same subfamily [10,11]. Nevertheless, reporter gene activities or *in situ* hybridization have revealed aquaporin expression in virtually all cell types examined [2]. Aquaporins are subject to regulatory mechanisms that possibly involve post-translational modifications. For instance, reversible phosphorylation controls the trafficking of mammalian aquaporin-2 (AQP2) to the apical membrane of renal-collecting-duct epithelial cells [12]. In spinach leaf PM the phosphorylation of PM28A (plasma membrane 28 kDa band) on Ser-274 is under the control of both calcium and apoplastic water potential [13]. A role for phosphorylation in plant aquaporin gating has been proposed, based on heterologous expression experiments in *Xenopus* oocytes [14,15]. Aquaporins can be subjected to other post-translational modifications including glycosylation [16] and deamidation [17], but their functional significance remains unclear.

In the present paper we have used tools developed for proteomics, i.e. separation of proteins by two-dimensional (2D) gel electrophoresis and protein identification by MS technology such as matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF MS) and electrospray-ionization tandem MS (ESI-MS/MS), to build an inventory of aquaporins expressed in the PM of *Arabidopsis* roots. In a preliminary analysis we showed that PIP2;2 is one of the most abundantly expressed aquaporin isoforms in this organ [18]. In this paper we provide evidence for

Abbreviations used: ESI-MS/MS, electrospray-ionization tandem MS; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; MIP, major intrinsic protein; NIP, nodulin-26-like intrinsic protein; PIP, plasma membrane intrinsic protein; PM, plasma membrane; TIP, tonoplast intrinsic protein; 2D, two-dimensional; DTT, dithiothreitol; PM28A, plasma membrane 28 kDa band.

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a high diversity of aquaporin expression and suggest, in addition, the presence of several types of post-translational modifications. This approach should provide clues to identifying novel molecular mechanisms for aquaporin regulation.

EXPERIMENTAL

Plant material

A. thaliana plants (ecotype Wassilewskija) were either wild-type or carried an *Agrobacterium tumefaciens* transferred DNA in the *PIP2;2* gene (*pip2;2-2* [18]). Plants were cultivated in hydroponic conditions in a growth chamber at 20 °C, with an 8 h light (150 $\mu E \cdot m^{-2} \cdot s^{-1}$)/16 h dark cycle at 70 % relative humidity. Plants were mounted on a 35 cm × 35 cm × 0.6 cm polystyrene raft floating in a basin filled with 8 l of culture medium [1.25 mM KNO_3, 0.75 mM $MgSO_4$, 1.5 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.1 mM Na_2SiO_3, 50 μM FeEDTA, 50 μM H_3BO_3, 12 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.7 μM $CuSO_4$, 1 μM $ZnSO_4$ and 0.24 μM MoO_4Na_2]. The culture medium was replaced weekly.

PM protein-extraction procedures

Roots were harvested 6 weeks after sowing. PM vesicles were purified from roots by aqueous two-phase partitioning, as described in [19], with the following modifications: root tissues were homogenized in a Waring blender for 5 × 10 s and PMs were purified from microsomes by partitioning in a mixture of polyethyleneglycol 3350/Dextran T-500, 6.4 % (w/w) each, in the presence of 5 mM KCl. Protein concentrations were estimated using a modified Bradford procedure [20]. The mean yield was 3.5 mg of protein/100 g of fresh weight. The sensitivity of the Mg^{2+}-dependent ATPase activity to vanadate, oligomycin and KNO_3 was used as enzymic markers of PM, mitochondria and tonoplast, respectively. The ATPase activities sensitive to vanadate, oligomycin and KNO_3 amounted to 93, 2.5 and 7.5 % of the total ATPase activity, respectively ($n = 3$). The contamination by Golgi membranes was assessed by inosine diphosphatase activity, which represented 4.2 % ($n = 3$) of the total ATPase activity. These results are similar to those obtained for various other PM-enriched fractions prepared by phase partitioning [21].

Extrinsic membrane proteins were stripped according to procedures described previously [22,23] with some modifications. Membranes (2 mg of proteins) were incubated in 40 ml of 5 mM EDTA, 5 mM EGTA, 4 M urea and 5 mM Tris/HCl, pH 9.5, for 5 min on ice before being centrifuged for 20 min at 100 000 ***g***. The subsequent pellet was resuspended in 20 mM NaOH and centrifuged at 100 000 ***g*** for 20 min. The membranes were then washed in 2 mM EDTA, 2 mM EGTA, 100 mM NaCl and 5 mM Tris/HCl, pH 8, centrifuged at 100 000 ***g*** for 20 min, and finally resuspended in 9 mM KCl, 300 mM sucrose, 5 mM Na_2EDTA, 5 mM Na_2EGTA, 50 mM NaF, 5 mM dithiothreitol (DTT), 2 μg/ml leupeptin and 10 mM Tris/borate, pH 8.3. The mean protein-purification yield was 20 %.

Gel electrophoresis

SDS/PAGE was carried out on 11 % acrylamide gels. Samples were resuspended in a Laemmli buffer at a temperature of < 25 °C to avoid irreversible aggregation. 2D gel electrophoresis was carried out according to [24] with slight modifications. Briefly, PM proteins were solubilized in 7 M urea, 2 M thiourea, 0.5 % Triton X-100, 1.2 % Pharmalytes (3–10), 20 mM DTT and 2 % (w/v) ASB14 (Calbiochem). Isoelectrofocalization was performed with a commercially immobilized pH gradient (linear pH gradient from 3 to 10, 18 cm length, Amersham Pharmacia Biotech), using a IPGphor apparatus (Amersham Pharmacia Biotech). The gel was rehydrated in the presence of the sample for 4 h without voltage and then for 7 h under constant voltage (50 V). The gel was then subjected to focusing until 80 kV · h was reached. After the isoelectrofocalization run, the first dimension gels were successively incubated at room temperature in solutions containing 2 % DTT and 2.5 % iodoacetamide according to [25]. The second dimension (SDS/PAGE) was carried out on homogenous 11 % T gels (Protean II; Bio-Rad). The first-dimension gels were sealed at the top of running gels with low-melting-point agarose [26]. Electrophoresis was conducted at 20 mA for 1 h, and then at 40 mA for 4–5 h. For analytical purposes or subsequent MS analysis, gels were stained with silver [25] or Coomassie Brilliant Blue G-250, respectively.

Western blotting

Western blotting was performed as described in [27] with slight modifications. Proteins were transferred on to Immobilon™ (Millipore) in liquid conditions (10 % methanol/10 mM Caps, pH 11, 16 h at 35 V). The blot was blocked for 1 h in a modified PBS containing 0.1 % (v/v) Tween-20 and 1 % BSA (PBSTB) [27], and incubated at room temperature (2 h) or at 4 °C (overnight) in the presence of the primary antibody (anti-PIP1;1 or anti-PIP2;2, diluted 1 : 5000). After washing (2 × 10 min) in PBSTB, the blot was incubated for 1 h with a peroxidase-labelled secondary antibody at 1 : 20 000 dilution in PBSTB. Secondary anti-chicken and anti-rabbit antibodies were used to detect the anti-PIP1;1 and anti-PIP2;2 primary antibodies, respectively. After washing (2 × 10 min) in PBS, the signal was revealed using a chemiluminescent substrate (Super Signal; Pierce).

Protein digestion

Electrophoretically separated proteins were excised from SDS/PAGE or 2D electrophoresis gels and washed successively for 30 min in 25 mM NH_4HCO_3, for 30 min in 50 % acetonitrile/25 mM NH_4HCO_3, and for 15 min with pure acetonitrile. Samples were then dried in a Speed-Vac centrifuge under vacuum. For SDS/polyacrylamide gels, reduction and alkylation of cysteine residues were accomplished as follows: gel pieces were incubated in 10 mM DTT/0.1 M NH_4HCO_3 for 45 min at 56 °C and then in 55 mM iodoacetamide/0.1 M NH_4HCO_3 for 30 min at room temperature in the dark and with shaking. The gel pieces were reswollen in the presence of 0.25–0.5 μg of sequencing-grade modified porcine trypsin (Promega, Madison, WI, U.S.A.) in 25 mM NH_4HCO_3 and incubated at 37 °C for 16 h. The supernatant of the tryptic digest was collected and the remaining peptides were extracted twice in 0.1 % trifluoroacetic acid/60 % acetonitrile by sonication for 15 min each. Supernatants were pooled and the final volume was reduced to 5 μl under vacuum. Protein digests were desalted using ZipTip μC_{18} columns (Millipore, Bedford, MA, U.S.A.) prior to MS analysis.

MS

The mass spectra were acquired with a Biflex III MALDI-TOF mass spectrometer (Bruker, Bremen, Germany) equipped with pulsed-ion extraction. The instrument was operated in the reflector mode. Trypsin-digested samples (0.8 μl) were mixed with 0.8 μl of matrix solution [α-cyano-4-hydroxycinnamic acid at

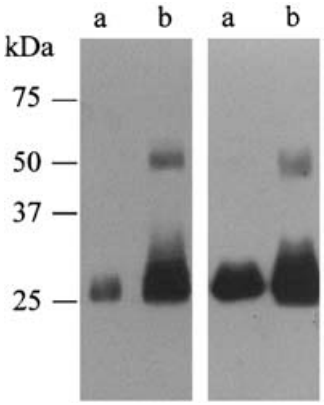

Figure 1 Immunodetection of PIPs in the PM of *Arabidopsis* roots

Total PM (**a**) and PM stripped with urea and NaOH (**b**) were separated by SDS/PAGE and probed with antibodies raised against PIP1;1 (right-hand panel; 5 μg of protein/lane) and PIP2;2 (left-hand panel; 10 μg of protein/lane).

half saturation in $1:1:2\times10^{-3}$ (by vol.) water/acetonitrile/trifluoric acid] and spotted on to the sample probe. The sample/matrix mixture was allowed to dry in air. Typically, 200 laser shots were averaged to produce one mass spectrum. The peptide mass fingerprint was subjected to Mascot software analysis (http://www.matrixscience.com). A score of >50 provides a statistically significant protein identification for *A. thaliana*.

MS/MS analyses were realized with a hybrid quadrupole/time-of-flight mass spectrometer (QTof II; Micromass, Manchester, U.K.) working in the nanoelectrospray ionization positive mode. We loaded 3–5 μl of each analysed desalted digest into a nanoelectrospray ionization needle (Protana/MDS, Odense, Denmark). The capillary voltage was set at 950 V, the cone voltage at 45 V, and collision energies were tuned to the order of 10–40 eV depending on the peptide mass and charge state. The instrument was calibrated in MS/MS mode with glufibrinopeptide. Argon was used as the collision gas. Instrument operation, data acquisition and analysis were performed using MassLynx/Biolynx 3.4 software (Micromass). MS/MS spectra were converted into DTA files and analysed using Mascot. Finally, each spectrum was interpreted manually to validate the identifications.

RESULTS

Several aquaporins are expressed in the root PM

A PM fraction was purified from *Arabidopsis* roots by two-phase partitioning. The presence of PIPs in this fraction was first evaluated using two antibodies raised against a 42-amino-acid N-terminal peptide of PIP1;1 and a 17-amino-acid C-terminal peptide of PIP2;2. Due to high sequence homology within the PIP1 and PIP2 groups, the anti-PIP1;1 and anti-PIP2;2 antibodies are expected to recognize PIP1;1–PIP1;4 and PIP2;1–PIP2;3, respectively. Using either antibody, a band at 26–29 kDa was detected in agreement with the calculated molecular mass of PIP1 and PIP2 homologues (Figure 1). Three procedures were compared for their efficiency to extract hydrophobic PM proteins. Purified PM was treated with 0.1 M Na_2CO_3, with 0.2 % Triton X-100 (w/v) [24] or with 4 M urea/20 mM NaOH (see the Experimental section). Samples were analysed by Western blot using the anti-PIP antibodies. We found that treatment of PM with urea and NaOH yielded the highest recovery of PIP proteins.

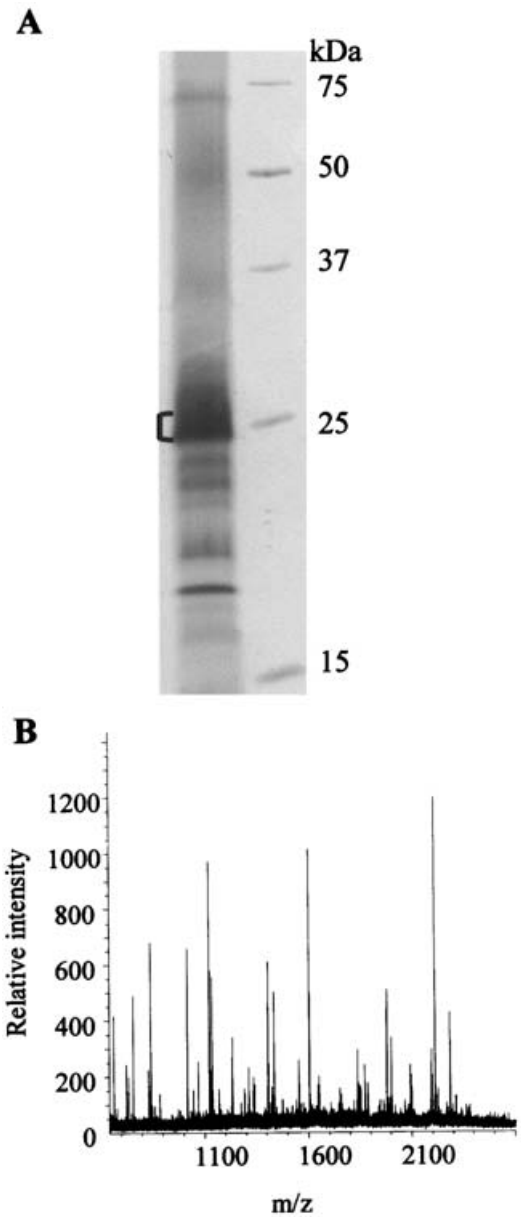

Figure 2 MALDI-TOF MS analysis of trypsin-cleaved 26–29 kDa band immunodetected by anti-PIP1;1 and anti-PIP2;2 antibodies in the root PM

(**A**) SDS/PAGE of PM stripped with urea and NaOH. The gel was loaded with 200 μg, electrophoresed and revealed by Coomassie Brilliant Blue G-250 staining. The bracket indicates the excised band. (**B**) MALDI-TOF MS spectrum showing the diversity of peptides detected.

Figure 1 shows that the PIP1 and PIP2 signals were clearly enhanced by this treatment. An additional signal at 52 kDa that can be assigned to previously described dimer forms of PIPs [28] was also detected in urea/NaOH-treated membranes.

The 26–29 kDa band immunodetected by the anti-PIP1;1 and anti-PIP2;2 antibodies (Figure 2A) was digested by trypsin and subsequently analysed by MALDI-TOF MS. A fingerprint with up to 43 peptides was obtained (Figure 2B) [18]. Analysis using the Mascot software allowed, with a statistically significant score of 64, the prediction of the presence of PIP2;1, six peptides of the fingerprint being matched to the PIP2;1 sequence [18]. The putative presence of other PIPs and more generally of all other *Arabidopsis* aquaporins was investigated by comparing

Table 1 Characterization by MALDI-TOF MS and ESI-MS/MS of putative aquaporin peptides detected in root PM

The masses $[M + H]^+$ of peptides that were identified from the 26–29 kDa fraction and that possibly correspond to a tryptic digest of aquaporin homologues are indicated in the first column. The corresponding sequence, isoform name and position in the sequence are also indicated. The sequence of certain peptides (third column) was determined by ESI-MS/MS (see text).

Measured peptide mass (Da)	Predicted peptide sequence	Determined sequence	Isoform	Position in the sequence
886.5*	KWSFYR		PIP2;1/PIP2;6	34–39/33–38
1017.5	VGANKFPER		PIP1;1/PIP1;2/PIP1;4	10–18
1069.5*	SFGAAVIYNK	SFGAAVI/LYNK	PIP2;1/PIP2;2	232–241
1122.5*	AFQSSYYTR	AFQSSYYTR	PIP2;1/PIP2;4	145–153
1136.5*	AFQSSYYDR	AFQSSYYDR	PIP2;2	143–151
1232.6	ALGSFGSFGSFR		PIP2;4	277–288
	QPIGTSAQSTDK		PIP1;4	19–30
1234.6*	DVEGPEGFQTR		PIP2;2	4–14
1292.7	SLGAAIIYNKDK		PIP2;5	231–242
1312.6*	SFGAAVIYNNEK	SFGAAVI/LYNNEK	PIP2;4/PIP2;7/PIP2;8	232–243/225–236/223–234
1340.6*	DLDVNESGPPAAR		PIP2;4	4–16
1404.7*	DVEAVPGEGFQTR	DVEAVPGEGFQTR	PIP2;1	4–16
1835.9	QYQALGGGANTVAHGYTK		PIP1;1	157–174
1872.9*	DYQDPPPAPFIDGAELK	DYQDPPPAPFI/LDGAELK	PIP2;1	17–33
1885.9	TPYNTLGGGANTVADGYSK	tag YNTLGG	PIP2;7	142–160
1960.9†	DYKEPPPAPFFEPGELK	tag APFFE	PIP1;5	31–47
1980.0	NIAIGGVQEEVYHPNALR	tag I/LAI/LGGV	TIP1;2	5–22
2000.9*	DYQDPPPAPFIDGAELKK	DYQDPPPAPFI/LDGAEL/IKK	PIP2;1	17–34
2067.0*	DYKDPPPAPFFDMEELR		PIP2;4	17–33
2096.9*†	DYEDPPPTPFFDADELTK	DYEDPPPTPFFDADEL/ITK	PIP2;2	15–32
2283.1	LMFKVTPEAFFGTTPADSPAR		NIP4;1	140–160
2296.2	VVDQEAGSTPSTLRDEDHPSR		NIP7;1	9–29
2363.1	EPPPAPFFEPGELSSWSFWR	EPPPAPFFEPGEL/ISSWSFWR	PIP1;1	33–52
2378.1	GFQPNPYQTLGGGANTVAHGYTK		PIP1;3	152–174

* Peptides sequenced previously [18].

† Peptides for which the ESI-MS/MS spectra are shown in Figure 3.

the theoretical fingerprint of each aquaporin, as calculated using the software PeptideMass (http://us.expasy.org/tools/peptide-mass.html), with the experimental fingerprint. Surprisingly, more than half (23) of the experimentally detected peptides could possibly be attributed to aquaporins (Table 1). These results extend a preliminary study performed on PIP2 homologues [18]. Thus putative fragments for all 13 PIPs, except PIP2;3, for one TIP and two NIPs could be predicted (Table 1).

To confirm some of the previous predictions, 12 of the 23 peptides assigned to aquaporins were partially sequenced by ESI-MS/MS and were identified by this technique (Table 1). Examples of MS/MS fingerprints are given in Figure 3. In all cases, the deduced amino acid sequence was in agreement with the predicted sequence. These results allow unambiguous identification of PIP1;1, PIP1;5, PIP2;1, PIP2;2, PIP2;7 and TIP1;2 in the 26–29 kDa fraction. More peptide sequences would be needed to establish unambiguously the presence of some other isoforms such as PIP1;3, PIP2;4, PIP2;5, NIP4;1 and NIP7;1. Due to a high similarity between PIP sequences, certain peptides can also match with several distinct PIP isoforms. For instance the peptide of 1069.5 Da can be assigned to PIP2;1 or PIP2;2 and the peptide of 1312.6 Da can be assigned to PIP2;4, PIP2;7 or PIP2;8 (Table 1).

PIP isoforms are expressed under several modified forms

The variety of PIP isoforms expressed in root PM was also evaluated by 2D gel electrophoresis. This technique allows the separation of closely related proteins provided that they differ in their isoelectric point (pI). The antibody raised against PIP1;1 can recognize four PIP1 homologues but surprisingly immunodetected at least seven spots (Figure 4A). A similar result was obtained with the anti-PIP2;2 antibody, which can recognize three PIP2 homologues (Figure 4B) and yet revealed at least six distinct spots. These results suggest that modified forms of PIP1 and PIP2 homologues, in addition to unmodified ones, are present in the root PM sample. Several of the immunodetected spots could be assigned to proteins revealed by silver-stained gel (Figure 4C).

Recently we characterized a *PIP2;2*-knockout line (*pip2;2-2* [18]) and this material was used to investigate which of the immunodetected spots were due to PIP2;2. PM proteins of roots from wild-type and *pip2;2-2* mutant plants were separated by SDS/PAGE. A similar band was detected with the anti-PIP2;2 antibody in the 26–29 kDa range in both wild-type and mutant PM (Figure 5A), suggesting that significant expression of PIP2;1 and PIP2;3 occurs in the two genotypes. Comparison of the 2D patterns revealed with the anti-PIP2;2 antibody in wild-type and mutant PM showed, however, that the most basic spot ($pI = 10$) was lacking in the mutant (Figure 5B). Thus the corresponding protein can be assigned to PIP2;2.

Proteins separated by 2D gel electrophoresis were revealed by Coomassie Brilliant Blue staining and those spots that were immunodetected by the anti-PIP antibodies were analysed by MALDI-TOF MS (Figure 4C). Fingerprints containing a few peptides could be established for each of the six spots analysed. MS/MS fragmentation analysis would have been necessary to unambiguously identify these peptides but their low abundance prevented such an analysis. We noted, however, that all the detected peptides (Table 2) had masses that corresponded to those peptides detected previously in the 26–29 kDa band (Table 1). This is consistent with the fact that, although separated by either SDS/PAGE or 2D gel electrophoresis, proteins from the same sample and with similar mass were analysed in both cases. Thus six of the peptides identified from 2D gels corresponded to aquaporin peptides identified and previously sequenced from the

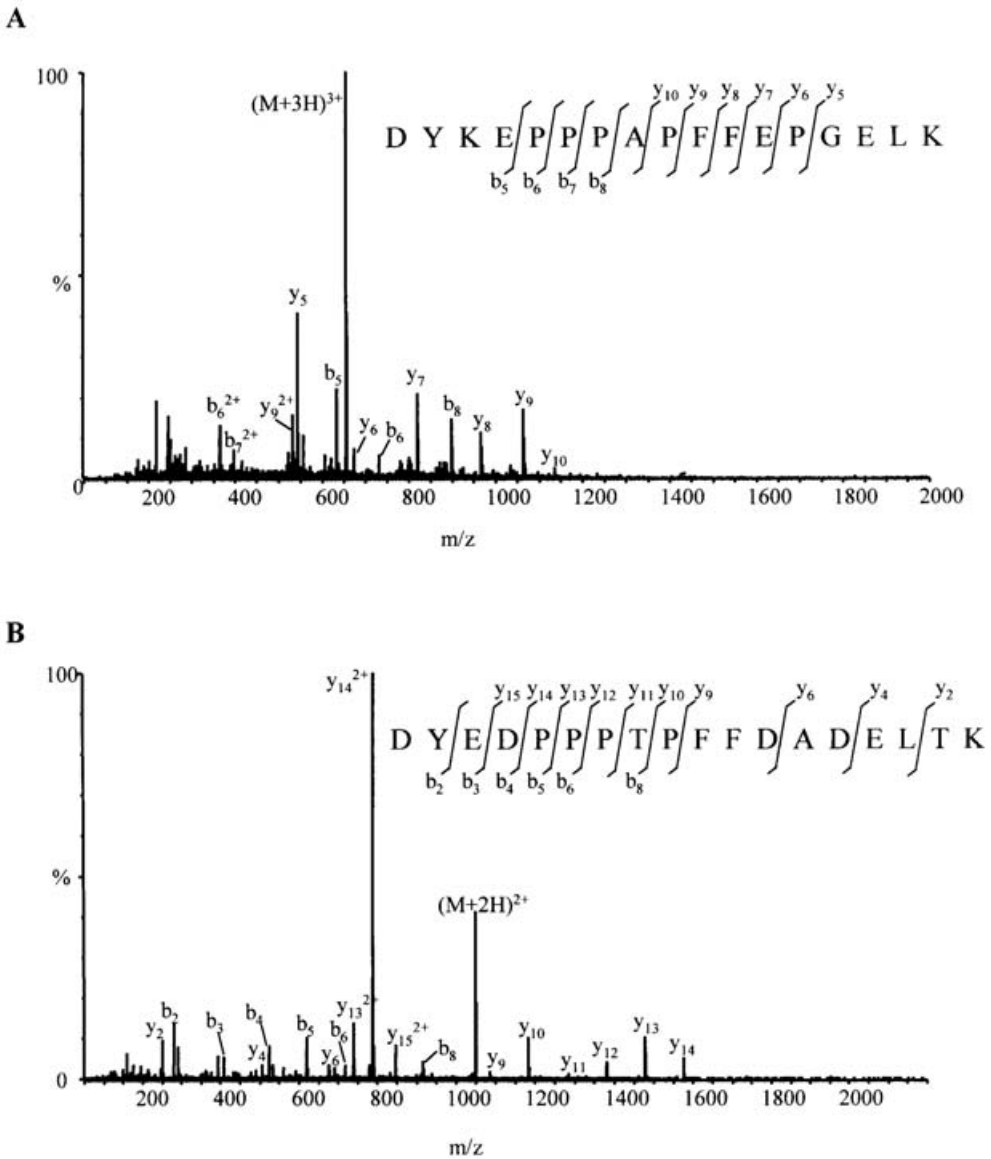

Figure 3 Sequence analysis of PIP1;5 (A) and PIP2;2 (B) peptides

Peptides were analysed by ESI-MS/MS. C-terminal (y-type) or N-terminal (b-type) fragments were used to interpret spectra. (**A**) Peptide (31–47) of PIP1;5. MS/MS analysis was performed on the $[M + 3H]^{3+}$ species of 654.3 Da that corresponds to the $[M + H]^{+}$ species of 1960.9 Da described in Table 1. A peptide sequence tag corresponding to the internal sequence Ala-Pro-Phe-Phe-Glu was deduced from y-type fragments in the spectrum. (**B**) Peptide (15–32) of PIP2;2. MS/MS analysis was performed on the $[M + 2H]^{2+}$ species of 1048.6 Da that corresponds to the $[M + H]^{+}$ species of 2096.9 Da described in Table 1. The whole sequence can be deduced from the spectrum.

26–29 kDa SDS/PAGE band (*m/z* 1069.5, 1122.5, 1136.8, 1404.7, 2000.9 and 2096.9; Table 2). Altogether, our data establish the presence of PIP2;1 in spots 1, 2, 3, 5 and 6 and the presence of PIP2;2 in spots 2, 3, 4, 5 and 6. The peptide of 1017.5 Da suggests the presence of at least one PIP1 isoform in spot 1 (Table 2 and Figure 4C).

The calculated pI of native PIP2;1 and PIP2;2 are 8.9 and 8.3, respectively [29]. Our data indicate, however, that isoelectrofocalization of these proteins can occur at pH between 5 and 9.8 for the most acidic and basic forms respectively (Figures 4 and 5B). Thus, post-translational modifications inducing a shift of pI may occur in both PIP2;1 and PIP2;2. Animal and plant aquaporins have been described as being phosphorylated [12–15]. We calculated that the addition of one phosphate would reduce the pI of PIP2;1 and PIP2;2 by 0.7 and 1.3 units, respectively. Furthermore, the addition of three phosphates should reduce the pI of PIP2;2 to 5. Thus some of the spots revealed at acidic pI values in Figures 4 and 5(B) might be accounted for by phosphorylation. The presence of phosphorylated PIP2;1 and PIP2;2 was investigated directly by MALDI-TOF MS analysis of the 26–29 kDa band. When used in the positive mode, MALDI-TOF MS analyses yielded fingerprints with one peptide (with a mass of 1617.82 Da) that could be assigned to a PIP1;1 or PIP1;2 peptide (QPIGTSAQSDKDYK) carrying one phosphate. When the negative mode was used, the overall number of peptides detected was reduced, but one novel peptide (746.3 Da) was revealed that could be assigned to a PIP2;1 or PIP2;2 peptide (SLGSFR) carrying one phosphate. The analysis in the negative mode requires a higher concentration of peptides. Such an analysis could not be engaged with 2D spots, which

Table 2 MALDI-TOF MS analysis of spots immunodetected by anti-PIP1;1 and anti-PIP2;2 antibodies in 2D gels of root PM proteins

The masses $[M + H]^+$ of peptides that were identified from 2D gel spots and that corresponded possibly to a tryptic digest of aquaporin homologues are indicated in the first column. The corresponding predicted sequence, isoform name and position in the sequence are also indicated. The last column refers to the spot numbers (see Figure 4) in which the peptides were identified.

Measured peptide mass (Da)	Predicted sequence	Isoform	Position in the sequence	Spot no.
1017.5	VGANKFPER	PIP1;1/PIP1;2/PIP1;3/PIP1;4	10–18	1
1069.5*	SFGAAVIYNK	PIP2;1/PIP2;2	232–241	1, 3–6
1122.5*	AFQSSYYTR	PIP2;1/PIP2;4	145–153	1–6
1136.5*	AFQSSYYDR	PIP2;2	143–151	2–6
1234.6	DVEGPEGFQTR	PIP2;2	4–14	2, 5, 6
1404.7*	DVEAVPGEGFQTR	PIP2;1	4–16	1–3, 5, 6
2000.9*	DYQDPPPAPFIDGAELKK	PIP2;1	17–34	6
2096.9*	DYEDPPPTPFFDADELTK	PIP2;2	15–32	6

* Peptides with masses that match peptides sequenced from SDS/PAGE (see Table 1).

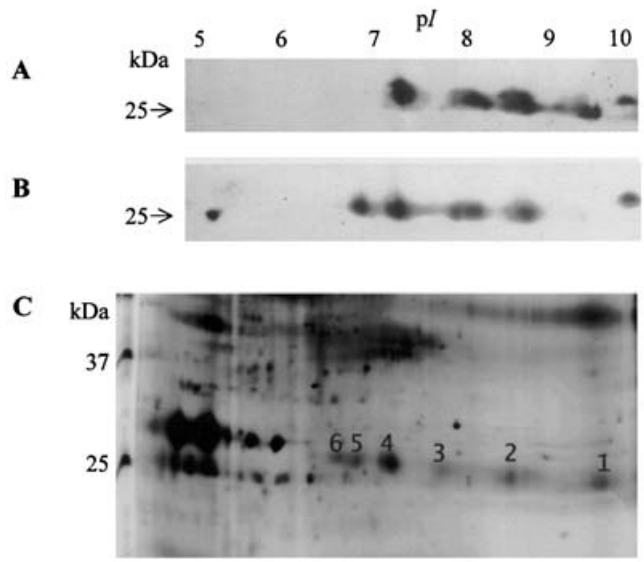

Figure 4 Separation of PIPs expressed in root PM by 2D gel electrophoresis

Root PM was purified and stripped with urea and NaOH as described in the Experimental section. Proteins separated by 2D gel electrophoresis were immunodetected by an anti-PIP1;1 antibody (**A**; 100 μg of protein on the gel) and an anti-PIP2;2 antibody (**B**; 50 μg of protein on the gel) or stained with silver (**C**). Numbers in (**C**) indicate spots that were analysed by MALDI-TOF MS (see Table 2).

yield lower amounts of peptides. The 746.3 Da peptide was fragmented tentatively by ESI-MS/MS (Figure 6). A major fragment, with a mass of 648.69 Da, was observed. It corresponds to a loss of 98 Da from the parent peptide. This was attributed to the loss of a phosphoric acid as H_3PO_4. Little or no additional fragmentation could be observed.

DISCUSSION

Antibodies have proven to be extremely useful to probe for the expression of specific proteins. Yet, in the case of aquaporins, and more generally of members of large gene families, a high degree of homology may prevent an efficient discrimination between close homologues. For instance, between PIP2;1 and PIP2;2 and between PIP2;2 and PIP2;3 there is 93.4% and 96.8% amino acid identity, respectively.

In the last 5 years MALDI-TOF MS and ESI-MS/MS have progressed into very sensitive and accurate techniques for characterizing peptides and proteins [30]. These techniques can provide an alternative to immunological approaches since they allow unambiguous identification of a protein of interest, even at a low abundance in a complex protein mixture. Most importantly, these techniques have the power to distinguish between highly homologuous proteins. In the present work we have used MS to establish an inventory of aquaporins expressed in the PM of *Arabidopsis* roots. Specific difficulties could be anticipated for such a study since (i) MS analysis can be hindered by the hydrophobicity of intrinsic membrane proteins and (ii) a large variety of similar aquaporin isoforms can be expressed in the same organ.

Aquaporins have been reported to be expressed abundantly in a variety of organisms, including plants. However, to detect aquaporin isoforms that might be poorly expressed, root PM preparations were enriched in intrinsic proteins by stripping membranes with urea/alkali treatment (Figure 1). Analysis by MALDI-TOF MS of the 26–29 kDa band that was strongly immunoreactive to anti-PIP1;1 and anti-PIP2;2 antibodies revealed up to 43 peptides. This suggested the presence of several co-migrating proteins in this sample. Nevertheless the PIP2;1 aquaporin could easily be identified. Due to their conserved structure, PIPs are thought to have similar biochemical properties, in terms of solubilization by detergents and accessibility to trypsin during digestion. Although MALDI-TOF MS analysis does not allow peptide quantification, we can nevertheless assume that PIP2;1 is one of the most abundant PIP isoforms in root PM [18].

Knowledge of sequences of all *Arabidopsis* aquaporins was very useful to investigate the presence of other isoforms. MS/MS analysis of peptides that could possibly be assigned to aquaporin homologues identified a total of five PIP isoforms unambiguously (PIP1;1, PIP1;5, PIP2;1, PIP2;2 and PIP2;7; Table 1). The peptides with *m*/*z* values of 1340.6, 1292.7 and 2378.1 Da allowed the prediction of the expression of three additional isoforms, PIP2;4, PIP2;5 and PIP1;3, respectively. Unfortunately, confirmation of the sequences of these peptides was prevented by their absence or low abundance in the ESI-MS/MS fingerprint.

The membrane sample used in this study was strongly enriched in PM, as assessed using enzymic markers (see the Experimental section). Overall, we found minimal contamination of PM by endomembranes. The main contamination arose from the vacuolar membrane (tonoplast), but this membrane did not contribute to more than 8% of the total ATPase activity. Consistent with this, only one TIP (TIP1;2) was identified that could be accounted for by the slight contamination of our PM preparation, or by the incidental presence of a TIP at the PM [31]. In contrast to TIP1;2, the PIP isoforms identified by MS/MS corresponded

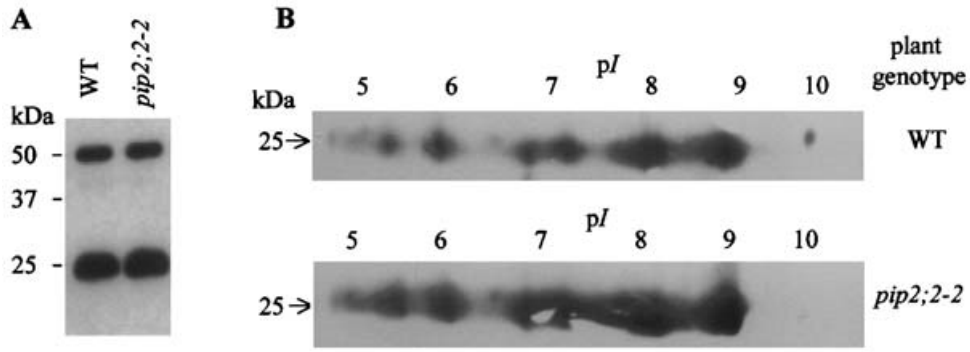

Figure 5 Comparative expression analysis of PIP2 isoforms in the root PM of wild-type (WT) and PIP2;2-knockout (*pip2;2-2*) plants [18]

Purified PMs were stripped with urea and NaOH. Proteins were separated by SDS/PAGE (**A**) or by 2D gel electrophoresis (**B**) and probed with an anti PIP2;2 antibody.

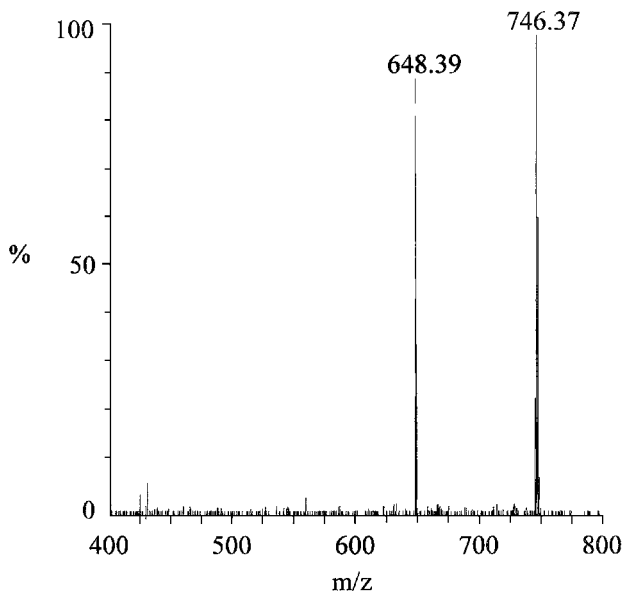

Figure 6 MS/MS analysis of a PIP2 phosphopeptide

The $[M+H]^+$ peptide of 746.37 Da was analysed by ESI-MS/MS. A major fragment of 648.39 Da is observed.

to abundant peptides, and thus it is likely that they are located at the PM. We also detected two putative peptides for NIP4;1 and NIP7;1. Consistent with a reduced number of expressed sequence tags [7], NIPs appear to be less abundantly expressed than PIPs and their subcellular localization remains as yet unknown. In the present work, we identified PIP peptides covering the N- and C-termini and loops C and E. A means to increase the sensitivity of aquaporin analysis by MS would be to improve the amino acid sequence coverage. Endoproteinase Lys-C, which has been used to analyse the sequence of the aquaporin PM28A, gave access to only one additional part within helix 6 [23]. Other enzymic or chemical types of cleavage should be tested for their ability to increase aquaporin sequence coverage.

Separation of proteins by 2D gel electrophoresis was used as another approach, complementary to MS, to investigate the diversity of PIPs in the root PM. A recently developed methodology for the solubilization and recovery of hydrophobic proteins on 2D gels [25,27] appeared imperative to resolve aquaporins by this technique. It was then possible to detect at least six spots with antibodies that recognized a total of three and four isoforms in the PIP1 and PIP2 subgroups, respectively. The use of a knockout PIP mutant was helpful in establishing that one spot was due exclusively to PIP2;2. The protein composition of the other spots was not established fully but MALDI-TOF MS analyses gave a first hint at a complex pattern with the presence of several homologues in the same spot and the same homologue being found in several spots.

Post-translational modifications could account for a situation where the number of spots in 2D gels was higher than the number of isoforms possibly detected. In particular, phosphorylation of proteins decreases their apparent pI and the modified forms can be resolved by 2D gel electrophoresis [32]. In the present work, MALDI-TOF MS analysis predicted the presence of phosphorylated forms in both the PIP1 (PIP1;1 or PIP1;2) and PIP2 (PIP2;1 or PIP2;2) subgroups. Phosphorylation of PIP1 would occur within a peptide that is located in the N-terminal part and contains a putative phosphorylation site (Ser-Asp-Lys). Phosphorylation of PIP2 homologues (PIP2;1 and/or PIP2;2) was suggested to occur at the C-terminal part of the protein. MS/MS analysis did not allow the identification of the phosphorylated residue but the loss of 98 Da, corresponding to the mass of H_3PO_4 from the parent peptide, suggests strongly that this peptide is phosphorylated (Figure 6). In addition, phosphorylation at a serine of the C-terminus has already been shown in PM28A, a PIP homologue of spinach leaf [13,15]. Identification of the phosphorylated peptide in spots from the 2D gels would have been of interest but was not possible because of low abundance.

In conclusion, the present paper demonstrates that proteomic tools can be used to analyse multigenic families of intrinsic membrane proteins. This is a critical advance since these proteins can be recalcitrant to separation by 2D gel electrophoresis and exhibit an extremely high sequence homology. Here we show that MS analysis and 2D gel electrophoresis provide novel insights into the diversity of aquaporin forms expressed in the PM of plant roots. Among the 13 PIPs encoded by the *Arabidopsis* genome, at least five (PIP1;1, PIP1;5, PIP2;1, PIP2;2 and PIP2;7) could be identified unambiguously. Most importantly, our results point to a high diversity of modified forms, including phosphorylated aquaporins. The identification of these modified forms will be instrumental in understanding novel molecular mechanisms of aquaporin regulation and their significance in water uptake by plant roots.

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